AD_____

Award Number: W81XWH-05-1-0255

TITLE: Specific, Reversible Cytostatic Protection of Normal Cells Against Chemotherapeutics in Breast Cancer Therapy

PRINCIPAL INVESTIGATOR: Benjamin B. Mull, OTHD

CONTRACTING ORGANIZATION: UT MD Anderson Cancer Center
Houston, TX 77030

REPORT DATE: March 2007

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	**2. REPORT TYPE**	**3. DATES COVERED** *(From - To)*
01/03/07	Annual Summary	21 Feb 2006 – 20 Feb 2007

4. TITLE AND SUBTITLE

Specific, Reversible Cytostatic Protection of Normal Cells Against Chemotherapeutics in Breast Cancer Therapy

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0255

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)
Benjamin B. Mull, OTHD

E-Mail: bmull@mdanderson.org

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

UT MD Anderson Cancer Center
Houston, TX 77030

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT: The adverse effects of cancer chemotherapy are widely recognized. Hair loss, gastrointestinal discomfort, lethargy and anorexia are quite common. The cause for these events is the nonspecific nature of current cancer treatment agents. Cytotoxic drugs, while effective at killing proliferating tumor cells, also target normal dividing cells. It is the purpose of this study to develop a proven in vitro strategy to protect normal dividing tissues using a cytostatic agent, UCN-01. There reversible arrest of normally dividing tissues in mice will be examined for improved tolerance of chemotherapeutics. This protective effect will also be evaluated in mice bearing orthotopically implanted breast tumors.

15. SUBJECT TERMS
Breast cancer, UCN-01, protection, cytostatic

16. SECURITY CLASSIFICATION OF:			**17. LIMITATION OF ABSTRACT**	**18. NUMBER OF PAGES**	**19a. NAME OF RESPONSIBLE PERSON**
a. REPORT	**b. ABSTRACT**	**c. THIS PAGE**			USAMRMC
U	U	U	UU	11	**19b. TELEPHONE NUMBER** *(include area code)*

Table of Contents

Introduction:

The side effects of cancer chemotherapy are well known. The purpose of this study is to determine if a protective protocol developed in cultured cells will be effective in an animal model system. UCN-01, a cytostatic drug and kinase inhibitor currently in preclinical trials, has been demonstrated in our laboratory to arrest normal cells but not tumor cells in a reversible fashion. The temporarily arrested normal cells are able to evade many of the toxicities of chemotherapeutic agents. The proposal reported upon here is to determine if UCN-01 can be used in a mouse model system to protect the normal dividing cells of the body from these toxic effects by placing them in a reversible state of arrest. The work in the first year of this project demonstrated that the dividing cells of the intestinal epithelium can be reversibly arrested by UCN-01. The work in the current award period focused on minimizing the antagonistic effect of the drug carrier (DMSO) on the action of UCN-01. Technical difficulties encountered during the tissue fixation procedure were also addressed and resolved.

Body:

During the first year of the award period, we demonstrated that UCN-01, a protein kinase inhibitor under development as a novel anticancer agent, was able to arrest the normally dividing cells of the small intestine, and that this effect was reversible. Small intestine was harvested from mice treated with UCN-01 by intraperitoneal administration and this demonstrated maximum cell cycle arrest approximately seven days following injection. Within four weeks of UCN-01 administration, these cell cycle kinetic parameters had returned to baseline. Furthermore, we demonstrated that the drug solvent, DMSO, resulted in an *increase* in the fraction of dividing cells in the control mice which were treated with DMSO alone. This was an unanticipated and undesired effect. Work during the most recent year of the award period was therefore focused on minimizing this effect of DMSO.

To address the issue of the undesired effect seen from the drug solvent, we first obtained a new aliquot of free (unsuspended) UCN-01 and dissolved it at a much higher concentration. Whereas the concentration in our original experiments was 4.8 mg/ml UCN-01 in DMSO, the newly obtained UCN-01 powder was dissolved at 19.2 mg/ml into DMSO. This higher concentration was designed to decrease the amount of DMSO an average 12-week old mouse would receive from approximately 30µl to less than 8µl, a four-fold decrease. An experiment identical to our original study was carried out. We used 3 cohorts of 10 mice each, planned for sacrifice at 1, 2 and 4 weeks following injection of the new UCN-01 suspension. We used a dose of 5mg/kg UCN-01 per animal, however this was in a lower volume due to the increased UCN-01 concentration. BrdU labeling and analysis were again performed on the small intestinal tissues of all mice. Unlike the result we noted in our initial experiments, the cell cycle arrest seen with 5mg/kg of UCN-01 at week one (Figure 1A) was not seen in the tissue samples from the new experiment (Figure 1B). Whereas the previous experiment showed a 40% reduction in cycling cells one week following UCN-01 injection, the intestinal tissues harvested from the mice in the new experiment demonstrated no significant change in cell cycle

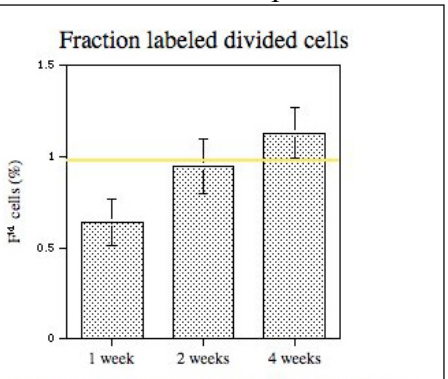

Figure 1A: Previously reported data indicating reversible cell cycle arrest in mice treated with 5 mg/kg UCN-01. Injected mice display a 40% decrease in dividing cells one week following treatment. This arrest abates between weeks two and four. The yellow line indicates the f^{1d} of normal (untreated) mice, 0.96%.

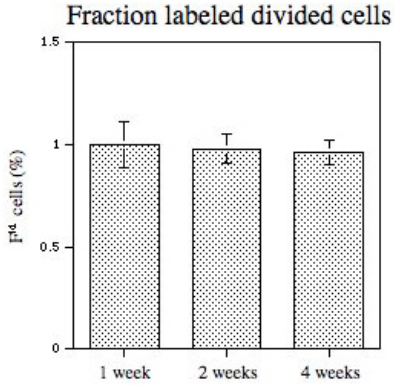

Figure 1B: Flow cytometric data obtained from mice receiving 5 mg/kg of concentrated (19.2mg/ml) UCN-01. Unlike the data shown in 1A, no arrest is seen at any time point.

kinetics. The fraction of labeled, dividing cells (those having been labeled by BrdU in S-

phase and successfully completed mitosis) in treated mice, was nearly the same as in control mice receiving only PBS (0.99% Fld). The 2 and 4 weeks samples provided similar values (Figure 1B). These data suggest that either the new UCN-01 powder used to create the higher concentration dose is inactive, or that an experimental error occurred.

To address the possibility of an experimental error, we first designed a study where we injected mice with the DMSO solvent alone. We had previously seen that DMSO has an effect which appears to counteract the UCN-01-mediated arrest. The higher concentration UCN-01 used in the previous experiment was intended to decrease this undesired effect of the DMSO solvent. Therefore, for this next experiment, DMSO volumes equivalent to those used with the 5mg/kg dose of UCN-01 were injected into 10 mice (7.5µl for a 30 mg mouse). After one week, the mice were injected with BrdU and sacrificed. The harvested intestinal tissue was prepared for flow cytometry analysis as in prior studies. It was hoped that the lower volume of DMSO would mitigate the increase in the fraction of labeled, divided cells that we had reported previously. It was expected that the lower volume of DMSO would induce less of an increase in the fraction of labeled, divided cells. Unfortunately, tissues harvested and fixed for this experiment were compromised, precluding meaningful analysis. Small particulate precipitates that interfered with the labeling and integrity of the DNA were seen in the ethanol/PBS fixed intestinal tissue. The DNA profiles obtained from this group had an unacceptable amount of sub-G1 content (Figure 2). A high sub-G1 content may be attributed to apoptosis; however, in these samples, no significant apoptosis was noted. A careful analysis of our experimental procedures and reagents isolated the cause of the precipitates. Previously, ethanol used in our laboratory had been shipped in plastic bottles. These had recently been replaced by 5-gallon metal containers. The metal containers impart an ionic component to the ethanol resulting in the precipitates seen in the ethanol/PBS fixative. These precipitates render the tissue samples unusable. We have recently returned to ordering ethanol in plastic bottles and this appears to have resolved the problem. Samples from untreated mice (solvent alone) using the corrected fixative had little sub-G1 content and a fraction of labeled, divided cells similar to previous data (0.99%).

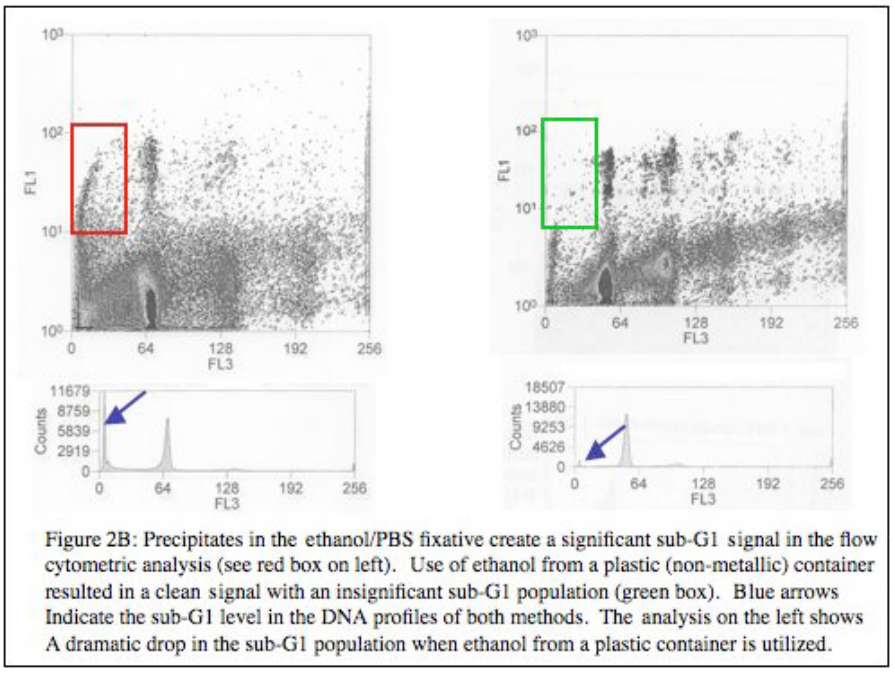

Figure 2B: Precipitates in the ethanol/PBS fixative create a significant sub-G1 signal in the flow cytometric analysis (see red box on left). Use of ethanol from a plastic (non-metallic) container resulted in a clean signal with an insignificant sub-G1 population (green box). Blue arrows Indicate the sub-G1 level in the DNA profiles of both methods. The analysis on the left shows A dramatic drop in the sub-G1 population when ethanol from a plastic container is utilized.

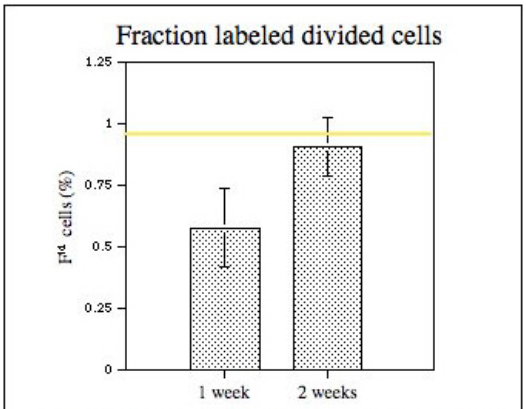

Figure 3: Mice treated with 5mg/kg new UCN-01 at the original concentration (4.8 mg/ml). Similar to the data in Figure 1A, flow cytometric analysis indicates a cell cycle arrest 1 week after treatment, and recovery by 2 weeks. The yellow line indicates the f^{1d} of normal (untreated) mice, 0.96%.

Having corrected the problem with our experimental technique, we next sought to determine whether the new drug was active. The new UCN-01 powder was suspended at the starting concentration of 4.8 mg/ml used in our initial experiments. The UCN-01 suspension was injected into 20 mice. 10 were labeled with BrdU and sacrificed after seven days. The other 10 mice were labeled and sacrificed at 2 weeks. Data obtained from flow cytometry analysis was similar to that seen previously with the original suspension; a decrease in the fraction of labeled, dividing cells at one week, and a recovery by the second week (Figure 3). These data suggest that the new UCN-01 powder is active. We therefore have repeated our experiment in which the UCN-01 is suspended at the higher concentration (19.2 mg/ml). Again, 30 mice were injected with 5 mg/kg of this suspension. Mice were BrdU-labeled and 10 were sacrificed at each of three timepoints; 1, 2 and 4 weeks. These samples have been harvested and, at the time of this submission we are in the process of analyzing the tissues for cell cycle kinetics.

Because of the finding that the DMSO solvent counteracts our drug (UCN-01) and due to the technical issues discussed above, a new timeline for the statement of work has been adopted:

Task 1. To demonstrate the arresting effects of UCN-01/staurosporine in mouse small bowel epithelium. (COMPLETED)

Task 2. To demonstrate reversibility of cytostatic effects and determine optimal time course for treatment. (COMPLETED; this includes the new DMSO experiments detailed in the narrative above)

a. Show ability of arrested cells to overcome cytostatic effects of agents (recovery shown at 5 mg/kg for one drug)
b. Determine length of effect for optimum doses from task 1
c. Optimize minimization of carrier (DMSO) effect

Task 3. To demonstrate improved tolerance of chemotherapeutics in mice receiving the arresting agents (Months 24-29)

a. Compare adverse effects of cytotoxic drugs in protected vs. unprotected mice

b. Increase doses of cytotoxic drugs in protected mice until adverse effects similar to unprotected. Creation of new MTD for protected mice

Task 4. To treat tumor-bearing mice with and without protection strategy (from task 3) to show improved treatment course (Months 28-36)

a. Optimize placement of orthotopic breast tumors in nude mice
b. Administration of i.v. chemotherapeutics to mice with and without receipt of protection protocol.

The timeline for Tasks 3 and 4 have been compressed from the original statement of work. To enable completion of the work, the experiments for Task 4 (treating tumor-bearing mice) will be initiated while the toxicology data from Task 3 is still being analyzed. The tumors will be implanted and will be ready to treat by month 29-30.

Key Research Accomplishments:

• Developed a strategy to minimize the undesired effects of the drug carrier (DMSO) on the action of UCN-01

• Resolved technical issue with ethanol component of tissue fixative

• Demonstrated activity of newly obtained UCN-01 in mouse model system

Reportable outcomes:

No reportable outcomes as of this writing.

Conclusions:

The arresting effect of UCN-01 on the normally dividing cells of the small bowel is counteracted by the drug's carrier, DMSO. A strategy to minimize this undesirable effect was developed using newly obtained UCN-01 in a greatly increased concentration, thereby decreasing the DMSO used in the mouse model system by four-fold. Technical issues encountered in the tissue fixation process were addressed and resolved. Finally, the activity of the newly obtained UCN-01 was confirmed. A current experiment using the more concentrated UCN-01 is expected to show a greater arrest of the normally dividing cells of the small bowel. Future experiments will use this new protocol to examine the effectiveness of the protection of normal tissues from chemotherapeutic toxicity.